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## Identification of Two Unknown Species of Bacteria

by Anna Albrecht

(Microbiology 1420)

### Introduction

**T**axonomy is defined as “the science that studies organisms in order to arrange them into groups (taxa)” (Nester, 2012). This seemingly simple description belies both the task of arrangement and the centuries of work that laid the foundation of the science. The science of taxonomy has grown from an artificial, imposed system of categorization based on gross physical characteristics to a highly sophisticated study of genetic evolution.

Carl von Linne, an 18<sup>th</sup> century Swedish physician, is considered by many to be the father of modern taxonomy (National Agricultural Library, n.d.). Linne is credited with developing an orderly system for naming and classifying plants based on reproductive structures, eventually publishing this work in *Species plantarum* (1753). His method of binomial nomenclature, using the genus and species as the scientific name of a particular organism, is still in use today.

Advances in Microbiology and Molecular Biology bring ever-increasing detail to the classification of microbial life. Gene mapping shows similarities between species at a molecular level, perhaps giving insight as to the evolutionary path of an organism. Carl Woese, a molecular biologist at the University of Illinois at Urbana, created controversy in the biology community when he announced his discovery that there exists a category of single-celled organisms distinct from prokaryotic and eukaryotic cell structure (Page, 1998). Woese argued that the then-contemporary method of classification, based on phenotype or even cellular structure, misses the most compelling information about organisms. By comparing the nucleic acid sequences of similar microorganisms, scientists can more accurately discern relatedness. He proposed the creation of a third taxon, called a Domain. He proposed the Domain Archaea as the third evolutionary lineage. (Woese, 1990)

Scientists now routinely use genetic sequencing to identify microbes, however, it is cost-prohibitive for most university microbiology labs to own such equipment. Microbiology students rely on observation of colony morphology, including size, shape, color and texture, to begin the process of identifying a microorganism. Microscopic examination, particularly after Gram stain or acid-fast stain reveals cell shape, size and staining characteristics. Biochemical testing, such as aerotolerance, further differentiates microbes based on optimal growth requirements. Other tests, including urea or gelatin hydrolysis measure the metabolic capabilities of an organism. Still others, such as nitrate reduction, test for the presence of end products to determine enzymatic pathways used by a microbe (Nester, 2012). By careful analysis of the requirements for cellular growth and metabolism, a microbe can be identified.

The purpose of the unknown lab is to apply the systematic reasoning learned in class to identify the prokaryotic bacteria in two unknown samples.

### Methods

The tests performed throughout the process of identifying unknown samples 16 A and 16 B were followed exactly as specified in the text *Microbiology: Laboratory Theory & Application* (Leboffe, 2008).

### **Gram Stain**

Both unknown cultures were tested using the Gram stain preparation. Gram staining is an important first step in differentiating bacteria into two distinct categories: Gram-positive and Gram-negative. The ability of a bacterium to retain the primary stain crystal violet is dictated by the structure of its cell wall. Gram-positive bacteria have a thick layer of peptidoglycan in their cell walls and are able to retain the primary stain. They appear violet when viewed under a microscope. Gram-negative cell walls have a thinner layer of peptidoglycan and a lipopolysaccharide layer and are unable to hold the primary stain. Gram-negative cells appear pink or red. In addition to providing information about cell wall structure, the Gram stain allows easy visualization of cell size, morphology (e.g. rod or bacillus) and arrangement (e.g. staphylococcal or streptococcal).

### **Nitrate Reduction Test**

The nitrate reduction test is used to differentiate bacteria based on their ability to use nitrate ( $\text{NO}_3$ ) as an electron acceptor and reduce it via anaerobic respiration. The ultimate product of nitrate reduction depends on the enzymatic pathway of the bacterium and its environment (Buxton, 2012). Certain bacteria use the enzyme nitrate reductase to reduce nitrate to nitrite ( $\text{NO}_2$ ). Others are capable of denitrification, a process whereby nitrate is reduced to molecular nitrogen ( $\text{N}_2$ ), a gas. The first step of the nitrate reduction test is to verify whether denitrification has occurred, as evidenced by the observation of a gas bubble in a Durham tube. If gas is present and the bacterium is a known non-fermenter, the test is positive for denitrification. If no bubble is observed, a second step is required to test for presence of nitrite ( $\text{NO}_2$ ). The test is positive if the broth turns red after reagents A and B are added.

### **Pigment**

Colony morphology, including pigment, is one of the simplest methods for differentiating bacteria. The pigment may be contained within the cells and thus color will be seen only within the colonies, however, some bacteria produce pigments that will diffuse into the medium. The intensity of the pigment may be influenced by temperature or the availability of nutrients. Some bacteria produce fluorescent pigment, which must be viewed under ultra-violet light.

### **Optimum Temperature**

Environmental temperature is a critical factor in bacterial growth. An organism can survive within a specific temperature range, but exhibits robust growth at an optimal temperature. Certain bacteria contain stable proteins that do not denature at higher temperatures (Nester, 2012). Most of the bacteria used in this unknown lab are mesophiles, with an optimal temperature range of  $25^\circ$  to  $45^\circ$  C. Only one possible bacterium is a thermophile, having an optimal temperature of  $55^\circ\text{C}$ . Incubating streak plates at varying temperatures and observing for absence of growth, size of colony formation or intensity of pigment may categorize bacteria according to temperature requirements for optimum growth (Leboffe, 2008).

**Results****Unknown Sample 16 A**

TEST	OBSERVATION	INTERPRETATION
Gram Stain	Rods of uniform shape and size; violet <i>and</i> pink cells observed	Indeterminate Gram Stain
Denitrification	No bubble in Durham tube	Negative for denitrification
Nitrate Reduction to NO <sub>2</sub>	Broth turned red after addition of Reagents A and B	Positive for nitrate reduction to NO <sub>2</sub>
Optimal Temperature: 37°C incubation	No growth	Negative for categorization as a mesophile
Optimal Temperature: 55°C incubation	Thick growth; small colonies that are opaque and dull beige in color.	Positive for categorization as a thermophile

**Unknown Sample 16 B**

TEST	OBSERVATION	INTERPRETATION
Gram Stain	Rods of uniform shape and size; violet <i>and</i> pink cells observed	Indeterminate Gram Stain
Denitrification	No bubble in Durham tube	Negative for denitrification
Nitrate Reduction to NO <sub>2</sub>	No color change of broth after addition of Reagents A and B	Negative for nitrate reduction to NO <sub>2</sub>
Pigment	Colonies glow when viewed under UV light	Positive for fluorescence

The first step in identification of unknowns 16 A and 16 B was to complete the Gram-stain procedure on each sample. Despite careful adherence to the steps outlined in Leboffe's manual, the test was inconclusive. Both samples were clearly identified as rods, having no particular arrangement, such as chain or paired groupings. Each slide contained a predominance of pink-to-red rods, however, violet stained cells were visible within both samples as well.

The next test performed was nitrate reduction. Unknown samples 16 A and 16 B were inoculated into nitrate broth and incubated according to standard procedure. Neither unknown sample produced gas, which would be evidenced by a bubble in the Durham tube, thus both 16 A and 16 B were negative for denitrification. Upon addition of Reagents A and B, only unknown 16 A exhibited color change of the broth to red. This indicates that the microbe 16 A is capable of reducing nitrate to nitrite and is interpreted as a positive test result; 16 B is negative for nitrate reduction to NO<sub>2</sub>.

Both samples were streak-inoculated onto TSA plates and incubated at 37°C. The purpose of this procedure was two-fold; first, to culture more of the bacteria in case the original sample was destroyed and second, to isolate colonies for observation of colony morphology. Unknown sample 16 B produced moderate growth, with isolation of shiny, opaque beige colonies. Interestingly, unknown sample 16 A produced no growth. A second plate was inoculated using unknown sample 16 A and incubated at 55°C. This incubation temperature yielded thick growth with isolation of small, opaque, dull beige colonies.

Streak plate 16 A showed no leaching of pigment into the medium, however, the colonies glowed when viewed under UV light.

## Discussion

The inconclusive results of the Gram stain test were disappointing. The procedure outlined in Leboffe was followed with careful attention paid to the timing of the decolorizing solution. Nevertheless, it is possible that the Gram positive sample was over-decolorized, or the Gram negative sample was under-decolorized, resulting in ambiguous results (Nester, 2012). The smears may have been applied too heavily, which can result in uneven exposure to reagents and mottled staining or the slide may have been overheated during fixation (Gram Variable Bacteria). Alternatively, the age of the unknown cultures may have affected the outcome. The cell walls in an older culture are known to be more susceptible to decolorization, particularly those in the genus *Bacillus* (Rao). This is a distinct possibility, especially given that the results of other tests performed on unknown sample 16 A support the conclusion that it is of the genus *Bacillus*. It is known that certain bacteria in the genus *Bacillus* produce a variable Gram stain result (Microbe Wiki).

The initial streak plate incubation – the most basic test – yielded clear results for unknown sample 16 A. When the culture failed to grow at 37°C, yet produced vigorous growth at 55°C, it was likely the sample was *Bacillus stearothermophilus*. This was supported by the results of the nitrate reduction test; the bacteria were negative for denitrification but positive for nitrate reduction to NO<sub>2</sub>. A number of tests, including Methyl Red, could have further supported the identification of unknown sample 16 A as *B. stearothermophilus*. According to Bergey's Manual of Determinative Bacteriology, *B. stearothermophilus* is Gram positive and a “chemoorganotroph, with a fermentative or respiratory metabolism” (1994, p. 559), so one could expect a positive Methyl Red test, as *B. stearothermophilus* is capable of mixed-acid fermentation.

Because *B. stearothermophilus* is a thermophilic, spore-forming bacterium, it can withstand extremes of heat and other environmental conditions. It is often used as a quality control measure to verify sterilization techniques in clinical or food preparation settings. *B. stearothermophilus* can cause spoilage of milk and other dairy products and is resistant to pasteurization. Fortunately, *B. stearothermophilus* is non-pathogenic and thus is a safe microbe to use as an indicator of faulty sterilization processes (Microbe Wiki).

Unknown sample 16 B was negative for denitrification and nitrate reduction to NO<sub>2</sub>, which indicates it is an aerobe or, in some instances, a facultative anaerobe. Since aerotolerance tests often yield variable results, that test was not performed at this time. Moderate growth of the microorganism on the streak plate incubated at 37°C indicates that the bacterium is most likely a mesophile. Because the media showed no evidence of pigment, *P. aeruginosa* was ruled out. Since the optimum growth temperature for a bacterium is closer to its maximum temperature, sample 16 A should have thrived at an incubation temperature of 37°C. However, growth was only moderate, indicating that perhaps its optimum temperature is lower than 37°C. Only two microbes in this lab have optimum temperatures below 37°C: *Serratia marcescens* and *Pseudomonas fluorescens*. According to the microbe chart provided prior to the lab, *S. marcescens* is capable of denitrification and was ruled out. Fluorescence was observed upon viewing unknown sample 16 B under UV light, confirming the bacteria as *Pseudomonas fluorescens*.

According to Bergey's Manual of Determinative Bacteriology, *P. fluorescens* is Gram negative and “Aerobic, having strictly respiratory type of metabolism with oxygen as its terminal electron receptor; in some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically” (1994, pp. 93 - 94). This variability in metabolism makes *Pseudomonas fluorescens* a resilient microorganism. It is abundant in nature, often present in soil and other organic matter. As part of a biofilm, it can cause food spoilage, especially of dairy products (Sillankorva, Neubauer, & Azeredo, 2008) and although it is non-pathogenic in a person with a healthy immune system, biofilm colonization of intravenous lines may cause infection in cancer patients (What is *Pseudomonas Fluorescens*?).

*Pseudomonas fluorescens* is a fascinating microorganism. It is capable of making an antibiotic known as mupirocin, which, by colonizing the root system of a plant, may resist fungal infection and increase nutrient absorption. As a mechanism for bioremediation, *P. fluorescens* is valuable for two reasons; first, it can breakdown a number of contaminants including plastic, and second, it can enable endemic plants to recover by colonization of root system (What is *Pseudomonas fluorescens*?).

The unknown lab exercise exemplifies how systematic examination of a sample using relatively simple, inexpensive tests can lead to reliable identification of a microorganism. Indeed, the simple streak plate incubation provided valuable information about colony morphology at different incubation temperatures as well as pigmentation. For a clinician with limited laboratory resources, colony or cell morphology may be the only test available and would dictate the plan of care; should a patient receive one category of antibiotic or another? Is an antibiotic even warranted?

While gene mapping is critical to the research and development of pharmacologic treatments, bioremediation, etc., it must not be considered more important than practical, mundane laboratory tests used to identify common bacteria. Pure science and applied science are symbiotic!

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